

## Relationship between Blood Levels of *N*-Carboxymethyl-lysine and Pentosidine and the Severity of Microangiopathy in Type 2 Diabetes

KUMIKO HIRATA AND KEIJI KUBO

*Department of Endocrine and Renal Medicine, Hiroshima Prefectural Hospital, 1–5–54, Ujinakanda, Minami-ku, Hiroshima 734-8530, Japan*

**Abstract.** The relationship between blood levels of *N*-carboxymethyl-lysine (CML) or pentosidine and the severity of microangiopathy was investigated in patients with type 2 diabetes. Blood CML and pentosidine levels were measured by ELISA in 97 type 2 diabetics (46 men and 51 women). CML and pentosidine levels were significantly higher in patients with chronic renal failure than in those with normoalbuminuria, microalbuminuria, or macroalbuminuria (all  $p < 0.05$ ). Among the diabetics without nephropathy ( $n = 49$ ), blood CML levels were significantly higher in the patients who had proliferative diabetic retinopathy than in those without retinopathy or those who had background retinopathy (both  $p < 0.01$ ). In contrast, blood pentosidine levels showed no significant differences among the three retinopathy groups. These findings suggest that the blood level of CML is related to the severity of both nephropathy and retinopathy, while the pentosidine level is only related to the severity of nephropathy.

**Key words:** Diabetic nephropathy, Diabetic retinopathy, *N*-carboxymethyl-lysine, Pentosidine

(*Endocrine Journal* 51: 537–544, 2004)

**ADVANCED** glycation end-products (AGE) form excessive cross-links between tissue proteins, thereby changing the structure of the extracellular matrix and altering the function of various cells via the AGE receptor. In diabetics, it has been shown that AGE levels increase in the blood and tissues prior to the onset of microangiopathy, and the AGE level has been reported to show a correlation with the progression of retinopathy and nephropathy [1–4]. More than 10 kinds of AGE have been identified to date, which differ with regard to their cross-links and fluorescence properties as well as the location and extent of their presence in various tissues [5, 6]. However, the role of each of these compounds in the onset and progression of diabetic complications has not been investigated in detail. In

this study, we measured the blood levels of two types of AGE, *N*-carboxymethyl-lysine (CML) and pentosidine, in patients with type 2 diabetes and assessed the relationship between these substances and the severity of diabetic retinopathy or nephropathy.

### Materials and Methods

The subjects were 97 inpatients of our hospital with type 2 diabetes mellitus (46 men and 51 women). On the day after admission, fasting blood samples were taken from the patients, and the fasting plasma glucose (FPG), hemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>), creatinine, CML, and pentosidine levels were measured. Height and body weight were also determined for calculation of the BMI. FPG and creatinine were measured by an enzymatic method, HbA<sub>1c</sub> was determined by high performance liquid chromatography (HPLC), and CML [7] and pentosidine [8] were measured by enzyme-linked immunosorbent assay (ELISA) as described below.

Received: October 20, 2003

Accepted: September 10, 2004

Correspondence to: Dr. Keiji KUBO, Department of Endocrine and Renal Medicine, Hiroshima Prefectural Hospital, 1-5-54, Ujinakanda, Minami-ku, Hiroshima 734-8530, Japan

## I. Measurement of CML

### *Pretreatment of serum samples*

Each specimen of blood (1 ml) was centrifuged at 3000 rpm for 5 min to separate it and after that was stored at  $-80^{\circ}\text{C}$  until use. To 100  $\mu\text{l}$  of serum diluted with 100 mM phosphate-buffered saline (PBS) pH 7.2, 100  $\mu\text{l}$  of 0.6% SDS/10 mM Tris-HCL saline (pH 7.4) and 5  $\mu\text{l}$  of 2 M  $\text{NaBH}_4$ /50 mM NaOH were added. The mixture was immediately heated to  $100^{\circ}\text{C}$  for 10 min. After cooling in ice water, 800  $\mu\text{l}$  of PBS was added and the sample was then used for assay of CML.

### *Raising the anti-AGE antibody*

AGE-keyhole limpet hemocyanin (KLH) was prepared by incubating 50 mg of KLH (CalBiochem, LaJolla, CA, USA) at  $37^{\circ}\text{C}$  for 16 weeks in 2 ml of sterile 0.4 M phosphate buffer (pH 7.2) containing 0.56 M glucose. Anti-AGE antibody was then raised in rabbits by interdermal injection of AGE-KLH (400  $\mu\text{g}$ ) emulsified with complete Freund's adjuvant, which was done 10 times at 4-week intervals. Antiserum obtained 2 weeks after the final injection was used for the assay.

### *Preparation of AGE-bovine serum albumin (BSA)*

AGE-BSA was prepared by incubating 5 g of BSA (Sigma, St Louis, MO, USA) at  $37^{\circ}\text{C}$  for 16 weeks in 30 ml of sterile 0.4 M phosphate buffer (pH 7.2) containing 0.56 M glucose. After dialysis against PBS, the AGE-BSA was stored at  $-80^{\circ}\text{C}$ .

### *ELISA*

A 96-well microplate (MaxiSorp, Nunc, Denmark) was coated overnight at  $4^{\circ}\text{C}$  with 100  $\mu\text{l}$  of AGE-BSA diluted  $10^5$ -fold with PBS. After washing with PBS containing 0.05% Tween-20 (PBST), 100  $\mu\text{l}$  of diluted ( $1:10^2$  to  $1:10^6$ ) AGE-BSA as the standard or serum treated as above, was added to each well. Then 100  $\mu\text{l}$  anti-AGE antiserum (diluted  $1:10^4$ ) was added and incubation was performed overnight at  $4^{\circ}\text{C}$ . After washing with PBST, 100  $\mu\text{l}$  of HRP-anti-rabbit immunoglobulin (Dako, Glostrup, Denmark) was added to each well and incubated for 4 h at  $25^{\circ}\text{C}$ . After washing again with PBST, 100  $\mu\text{l}$  of 3,3',5,5'-tetramethylbenzidine (ScyTek Laboratories, West Logan, UT, USA) solution was added to each well and incubation was done for 40 min. Finally, the optical density was measured at 450 nm with a microplate ELISA reader (Molecular

Devices, Sunnyvale, CA, USA) after addition of 50  $\mu\text{l}$  of 2 N  $\text{H}_2\text{SO}_4$ .

The anti-AGE antibody that was raised did not cross-react with Amadori products. Amadori products in the tested serum were reduced by  $\text{NaBH}_4$ /50 mM NaOH during pretreatment of serum samples, and therefore were not detected by our assay. Because our antibody mainly reacted with CML and only reacted weakly with pentosidine, AGE measured by this assay was thought to represent CML and AGE detected by the assay is called CML in this report. The intra-assay coefficient of variation of our ELISA was 4.8–10.2% and the inter-assay values were 3.5–6.2%.

## II. Measurement of pentosidine

### *Synthesis of pentosidine*

L-arginine (Sigma), L-lysine (Sigma), and D-ribose (Wako Pure Chemicals Industries, Osaka, Japan) were dissolved in phosphate buffer (pH 7.4) at 100 mM each, sterilized by filtration, and incubated at  $37^{\circ}\text{C}$  for 2 months. The reaction mixture was then loaded onto a column filled with Dowex 50W-X8 (Muromachi Kagaku Kogyo Kaisha, Tokyo, Japan), which was washed with distilled water and 1 M pyridine. After washing again with distilled water, the column was eluted with 2 N NaOH and the fractions collected were adjusted to pH 7.4 with hydrochloric acid. Next, the concentrated sample was loaded onto TSK gel (Toyo-pearl HW-40s; Tosoh, Tokyo, Japan) and eluted with distilled water while fractions were obtained using a fraction collector.

The fluorescent fraction (excitation at 335 nm and emission at 385 nm) was further purified by HPLC (Shimadzu, Kyoto, Japan) using a Develosil ODS-FG-5 column and a mobile phase of 5%  $\text{CH}_3\text{CN}$  and 0.1% trifluoroacetyl in water. The fraction collected was confirmed to be pentosidine by mass spectrometry and NMR spectrometry.

### *Conjugation of synthetic pentosidine with albumin*

Synthetic pentosidine was conjugated with BSA as follows. Pentosidine (15  $\mu\text{mol}$ ) was incubated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (0.1 M; Pierce Chemical, Rockford, IL, USA), N-hydroxysulfosuccinimide (1.4 mg, Pierce Chemical), and BSA (7 mg) in phosphate-buffered saline (PBS) at room temperature for 4 h and then overnight at  $4^{\circ}\text{C}$ . After this, the mixture was dialyzed against PBS.

### *Antibody production*

Pentosidine-BSA emulsified with complete Freund's adjuvant was injected into white rabbits. The first dose of 1.0 mg was injected subcutaneously into the back and four additional injections were given at 2-week intervals as boosters. Three days after the last injection, the rabbits were sacrificed and antipentosidine antiserum was obtained. The antiserum was loaded onto a pentosidine-immobilized, CNBr-activated Sepharose 4B column (Pharmacia, Uppsala, Sweden). After washing, the column was eluted with 4 M magnesium chloride while monitoring at OD 280, and the fraction obtained was dialyzed against PBS to obtain polyclonal rabbit antipentosidine antibodies. The purity of the antibodies was tested by denaturation in boiling water for 5 min in the presence of mercaptoethanol. Samples were then separated by SDS-PAGE using a 5–20% gradient gel, which was stained with Coomassie brilliant blue. The antibody raised against pentosidine did not recognize the raw materials used (L-arginine, L-lysine, and D-ribose) or compounds with similar structures (adenine and azabenzimidazole). These results suggested that the antibody was specific for pentosidine.

### *Pentosidine-KLH*

KLH (7.0 mg) was incubated with 1.5  $\mu$ l of pentosidine in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysulfosuccinimide in PBS for 4 h at room temperature and then overnight at 4°C.

### *Pretreatment of samples*

Enzymatic digestion was used to expose the pentosidine molecule bound to plasma proteins. Fifty microliters of pentosidine was added to 20  $\mu$ l of pronase (Calbiochem) and 80  $\mu$ l of Tris-HCL buffer and incubated at 55°C for 1.5 h. The mixture was then heated in boiling water for 15 min to inactivate the enzyme.

### *Competitive ELISA for pentosidine*

Pentosidine-KLH conjugate (100  $\mu$ l) was dispensed into each well of a microtiter plate and incubated for 24 h at 4°C. After washing with PBS containing 0.5 ml/l Tween 20, the wells were blocked with 40% Block Ace (Dainihon Pharmaceutical, Osaka, Japan) for 3 h at room temperature.

Fifty microliters of anti-pentosidine antibody and 50  $\mu$ l of pentosidine standard solution or pretreated plasma sample were added to each well and incubation

was done for 1 h at 37°C after washing. Peroxidase-labeled goat anti-rabbit polyclonal IgG antibodies were then added and incubation was done for 1 h at room temperature.

Next, a coloring reagent containing 0.5 mg/ml of 3,3',5,5'-tetramethylbenzidine was added to each well. The reaction was stopped after 10 min by adding 100  $\mu$ l of TMB stop buffer (ScyTek Laboratories) and the absorbance was measured within 10 min at 450 nm (main wavelength) and 630 nm (reference wavelength). A standard curve was created by measuring standard pentosidine solutions (0, 0.00005, 0.0005, 0.005, 0.05, 0.5, and 5.0  $\mu$ g/ml).

The subjects were divided into two groups according to their serum creatinine concentrations, with one group having a creatinine level of 1.3 mg/dl or more (the chronic renal failure [CRF] group,  $n = 6$ ) and the other having a level of 1.2 mg/dl or less (the non-CRF group,  $n = 91$ ). In the non-CRF group ( $n = 91$ ), a 24-hour urine specimen was collected and analyzed for albumin by the turbidimetric method. Based on the urinary albumin excretion, these 91 patients were then divided into three groups, which showed albumin excretion of less than 30 mg/day (normoalbuminuria [Normo] group,  $n = 49$ ), excretion of 30 to 300 mg/day (microalbuminuria [Micro] group,  $n = 24$ ), and excretion of 300 mg/day or more (macroalbuminuria [Macro] group,  $n = 18$ ). Based on the ophthalmoscopy findings, all 97 patients were divided into a group without diabetic retinopathy (NDR group,  $n = 42$ ), a group with background diabetic retinopathy (BDR group,  $n = 18$ ), and a group that had proliferative diabetic retinopathy (PDR group,  $n = 37$ ). Patients on maintenance hemodialysis and patients with complications of arteriosclerosis were excluded from the study. This study was approved by the Ethics Committee of Hiroshima Prefectural Hospital, and informed consent was obtained in writing from all participating patients.

Results are expressed as the mean  $\pm$  standard deviation. The significance of differences between groups was assessed by one-way ANOVA and the multiple comparison test, with  $p < 0.05$  indicating statistical significance.

## **Results**

The average age of the subjects was  $59.4 \pm 10.4$  years and the duration of diabetes was  $10.7 \pm 8.0$  years, while

FPG was  $173.6 \pm 56.4$  mg/dl, HbA<sub>1c</sub> was  $9.0 \pm 1.9\%$ , and BMI was  $24.3 \pm 4.0$  kg/m<sup>2</sup> (Table 1). Diabetes was treated by diet and exercise alone in five patients, oral hypoglycemic agents in 58 patients, and insulin therapy in 34 patients.

The blood level of CML was not correlated with that of HbA<sub>1c</sub> ( $r = 0.041$ ,  $p = 0.701$ ) and the blood pentosidine level was also not correlated with that of HbA<sub>1c</sub> ( $r = -0.088$ ,  $p = 0.385$ ). Furthermore, the blood level of CML was not correlated with urinary albumin excretion ( $r = -0.019$ ,  $p = 0.850$ ) and neither was the blood level of pentosidine ( $r = 0.121$ ,  $p = 0.294$ ). The blood CML level of the healthy control subjects ( $2.05 \pm 0.40$  mU/ml,  $n = 10$ ) was not significantly different from that of the Normo group or the NDR group. In addition, the blood pentosidine level of healthy control subjects ( $0.043 \pm 0.010$   $\mu$ g/ml,  $n = 20$ ) was not significantly different from that in the Normo group or the NDR group.

The relationship between the progression of nephropathy and the blood level of CML was investigated in all 97 patients. It was found that CML levels were significantly higher in the CRF group ( $4.32 \pm 2.09$  mU/ml) than in the Normo, Micro, and Macro groups ( $2.02 \pm 0.54$ ,  $2.09 \pm 0.76$ , and  $2.27 \pm 0.64$  mU/ml, respectively; all  $p < 0.01$ ) (upper panel of Fig. 1). The relationship between progression of nephropathy and blood pentosidine levels was also assessed in all 97 patients. Pentosidine was significantly higher in the CRF group ( $0.175 \pm 0.173$   $\mu$ g/ml) than in the Normo and Micro groups ( $0.061 \pm 0.025$  and  $0.073 \pm 0.036$   $\mu$ g/ml, respectively; both  $p < 0.01$ ) or the Macro group ( $0.098 \pm 0.113$   $\mu$ g/ml,  $p < 0.05$ ) (lower panel of Fig. 1).

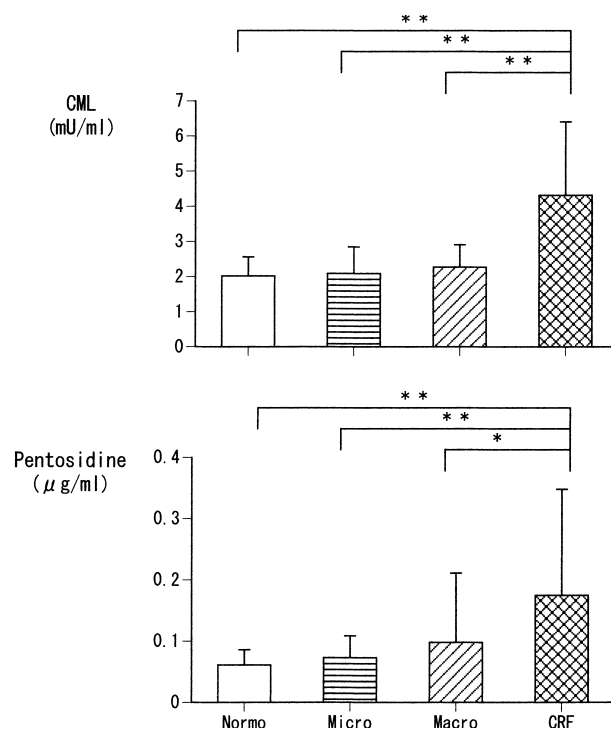
When the relationship with retinopathy was similarly explored in all 97 patients, blood levels of CML were significantly higher in the PDR group ( $2.58 \pm 1.20$  mU/ml) than in the NDR group ( $1.98 \pm 0.55$  mU/ml) ( $p < 0.01$ , upper panel, Fig. 2). In contrast, no significant difference was found between the NDR group and the BDR group ( $2.09 \pm 0.87$  mU/ml) or between the BDR and PDR groups. Blood pentosidine levels were also significantly higher in the PDR group ( $0.098 \pm 0.108$   $\mu$ g/ml) than in the NDR group ( $0.063 \pm 0.028$   $\mu$ g/ml) ( $p < 0.05$ , lower panel, Fig. 2), but showed no significant differences between the NDR group and BDR group ( $0.071 \pm 0.042$   $\mu$ g/ml) or between the BDR and PDR groups.

To exclude the influence of nephropathy, the 49 patients without any evidence of nephropathy were ex-

**Table 1.** Clinical characteristics of the subjects

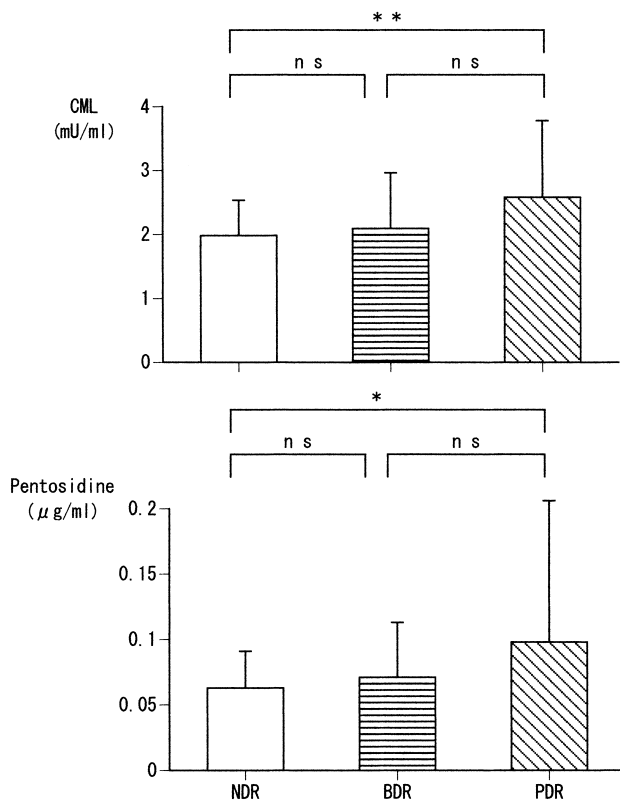
Number of subjects (Male/Female)	97 (46/51)
Age (years)	$59.4 \pm 10.4$
Duration (years)	$10.7 \pm 8.0$
FPG (mg/dl)	$173.6 \pm 56.4$
HbA <sub>1c</sub> (%)	$9.0 \pm 1.9$
BMI (kg/m <sup>2</sup> )	$24.3 \pm 4.0$

FPG: fasting plasma glucose; BMI: body mass index  
Values are given as the mean  $\pm$  SD.

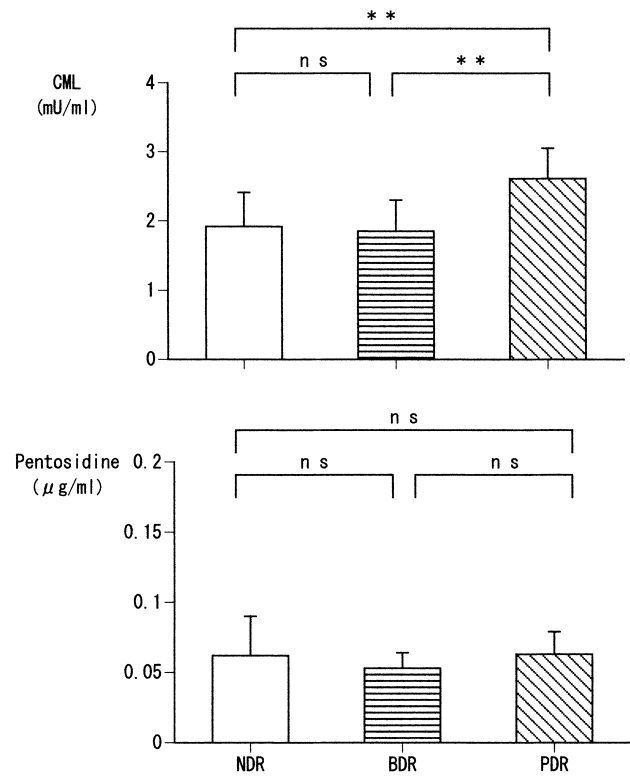


**Fig. 1.** Relationship of the blood levels of CML and pentosidine to the severity of diabetic nephropathy in all 97 subjects. Normo: Normoalbuminuria; Micro: Microalbuminuria; Macro: Macroalbuminuria; CRF: Chronic Renal Failure. \*\*  $p < 0.01$  vs CRF. \*  $p < 0.05$  vs CRF.

amined separately to assess the relationship between progression of retinopathy and the blood CML level. The PDR group ( $n = 8$ ,  $2.61 \pm 0.44$  mU/ml) showed a significantly higher blood level of CML than the NDR group ( $n = 35$ ,  $1.92 \pm 0.49$  mU/ml) or the BDR group ( $n = 6$ ,  $1.85 \pm 0.45$  mU/ml) (both  $p < 0.01$ , upper panel, Fig. 3). In contrast, there was no significant difference between the NDR and BDR groups. When the relationship between the progression of retinopathy and the blood pentosidine level was assessed in the 49 patients



**Fig. 2.** Relationship of the blood levels of CML and pentosidine to the severity of diabetic retinopathy in all 97 subjects. NDR: No Diabetic Retinopathy BDR: Background Diabetic Retinopathy PDR: Proliferative Diabetic Retinopathy \*\*  $p < 0.01$  vs PDR. \*  $p < 0.05$  vs PDR.



**Fig. 3.** Relationship of the blood levels of CML and pentosidine to the severity of diabetic retinopathy in 49 patients without nephropathy. NDR: No Diabetic Retinopathy BDR: Background Diabetic Retinopathy PDR: Proliferative Diabetic Retinopathy \*\*  $p < 0.01$  vs PDR.

without nephropathy, there were no significant differences among the NDR ( $0.062 \pm 0.028 \mu\text{g/ml}$ ), BDR ( $0.053 \pm 0.011 \mu\text{g/ml}$ ), and PDR groups ( $0.063 \pm 0.016 \mu\text{g/ml}$ ) (lower panel, Fig. 3).

## Discussion

In diabetics, binding of AGE to collagen in the vessel walls and skin can already be detected at the stage of microalbuminuria or minimal change retinopathy, and such binding of AGE has been reported to increase with the progression of diabetic complications [1, 2, 9]. Furthermore, the circulating level of AGE in the blood has been reported to show a correlation with the histological and clinical severity of nephropathy [4]. In the present study, we investigated whether the circulating levels of CML and pentosidine (two types of AGE)

showed any relationship with the severity of nephropathy or retinopathy.

Although the blood levels of CML and pentosidine tended to increase with the progression of nephropathy, no significant differences were seen among the Normo, Micro, and Macro groups. A significant increase of CML and pentosidine was only noted after the onset of CRF along with the elevation of serum creatinine. Since CML and pentosidine are eliminated by renal excretion, their blood levels are directly proportional to the creatinine level and are inversely proportional to creatinine clearance [7, 8]. Therefore, the present results reflected the fact that the blood levels of CML and pentosidine are influenced by renal clearance. Moreover, both glycosylation and oxidation are essential for the formation of CML and pentosidine [10–12]. An increase of oxidative stress is observed in diabetics with microalbuminuria and this becomes more marked after

the onset of uremia. Accordingly, the increased oxidative stress associated with CRF may accelerate the formation of CML and pentosidine.

In our study, the blood levels of CML or pentosidine were not correlated with the stage of diabetic nephropathy and were also not correlated with urinary albumin excretion. Pentosidine levels in the skin, but not serum, were reported to show a correlation with severity of complications in patients with type 1 diabetes [13]. Furthermore, CML and pentosidine have been demonstrated to accumulate in the expanded mesangial matrix and nodular lesions in diabetic nephropathy [14]. These observations suggest that tissue, but not serum, levels of glycosidation products such as CML and pentosidine may be used a marker for the development of diabetic nephropathy.

In the present study, the blood levels of both CML and pentosidine were not correlated with HbA<sub>1c</sub>. Takeuchi *et al.* reported that blood CML levels were not associated with HbA<sub>1c</sub> in patients with type 2 diabetes [15], while Sanaka *et al.* found that blood pentosidine and blood glucose levels were not correlated [8]. These reports are consistent with our findings, suggesting that the production of CML or pentosidine does not directly reflect the severity of hyperglycemia.

In relation to diabetic retinopathy, blood levels of CML and pentosidine showed no significant difference between the NDR and BDR groups, but were significantly higher in the PDR group than the NDR group. Among the 37 patients with PDR, 29 also had nephropathy and were classified into the Micro or Macro group. Thus, the increase of blood CML and pentosidine levels noted in the PDR group is likely to have been influenced by the decline of renal clearance in these 29 patients. The relationship between diabetic retinopathy and CML or pentosidine was also examined in diabetics without any nephropathy (*i.e.*, patients classified into the Normo group). Blood CML levels were significantly higher in the patients with PDR than in those with NDR or BDR, showing that the blood CML level increased along with the progression of retinopathy before the onset of microalbuminuria. In the case of pentosidine, however, the blood level did not show any increase with the progression of retinopathy.

Endo *et al.* studied the proliferative membranes removed from patients with proliferative diabetic retinopathy, and reported that CML was diffusely deposited around small blood vessels and in the glial cells of these membranes [16]. In view of these findings, it

seems possible that the processes of glycosylation and oxidation participate in the onset and progression of diabetic retinopathy, and it can be strongly suggested that CML plays an important role in the progression of retinopathy. However, no reports have been published on the histological localization of pentosidine, and so when and how pentosidine participates in the onset and progression of retinopathy remains unclear in many respects. It has been reported that the accumulation of pentosidine in skin biopsy specimens is directly proportional to the severity of diabetic retinopathy [13, 17, 18], but it has also been reported that there is no significant correlation between pentoside accumulation and the early minimal changes of retinopathy [2]. The present study showed that blood CML levels had a significant relationship with the severity of retinopathy in diabetics without any nephropathy, whereas pentosidine levels had no relationship with retinopathy. Accordingly, CML is more likely to be involved in the progression of diabetic retinopathy than pentosidine.

Histological examination of renal lesions has shown that CML is the principal compound of AGE in the mesangium, basement membrane, and vessel walls, while pentosidine shows relatively little deposition in these regions although it is widely distributed [19]. The same study also revealed that the level of AGE receptor mRNA expression provoked by CML in the mesangium and basement membrane is correlated with the severity of glomerulosclerosis, whereas the response to pentosidine shows no such correlation [19]. These histopathological findings suggest that CML plays an important role in the onset and progress of microangiopathy, and that an increase of oxidative stress accelerates CML formation. Since chronic treatment with AGE-BSA was reported to induce glomerular sclerosis and albuminuria in normal rats [20], various forms of AGE such as CML or pentosidine may participate in the progression of diabetic nephropathy in patients with type 2 diabetes. Further studies will be required to define a cause-and-effect relationship between CML or pentosidine and diabetic nephropathy in humans, including intervention studies using AGE inhibitors.

Kiuchi *et al.* reported that AGE levels were increased in type 2 diabetic patients with coronary artery disease [21]. Since patients with arteriosclerotic complications were excluded from our study, such complications had no influence on the blood levels of CML or pentosidine. Because diabetic patients with arteriosclerosis often have microangiopathy as well, it is diffi-

cult to distinguish an increase of CML or pentosidine due to microvascular complications from an increase due to arteriosclerotic complications. Therefore, the relationship between CML or pentosidine and arteriosclerosis in diabetic patients needs to be clarified in the future.

Further studies are needed to examine whether or not

the blood levels of various forms of AGE accurately reflect the severity of tissue lesions during the course of microangiopathy. If blood AGE levels prove to be useful markers, this may make it possible to assess the severity of complications earlier as well as monitoring the response to therapy, suggesting that these markers may be of high clinical relevance.

## References

1. Makita Z, Radoff S, Rayfield EJ, Yang Z, Skolnik E, Delaney V, Friedman EA, Cerami A, Vlassara H (1991) Advanced glycosylation end products in patients with diabetic nephropathy. *N Engl J Med* 325: 836–842.
2. Beisswenger PJ, Makita Z, Curphey TJ, Moore LL, Jean S, Brinck-Johnsen T, Bucala R, Vlassara H (1995) Formation of immunochemical advanced glycosylation end products precedes and correlates with early manifestations of renal and retinal disease in diabetes. *Diabetes* 44: 824–829.
3. Vlassara H, Palace MR (2002) Diabetes and advanced glycation endproducts. *J Intern Med* 251: 87–101.
4. Berg TJ, Bangstad HJ, Torjesen PA, Osterby R, Bucala R, Hanssen KF (1997) Advanced glycation end products in serum predict changes in the kidney morphology of patients with insulin-dependent diabetes mellitus. *Metabolism* 46: 661–665.
5. Onorato JM, Thorpe SR, Baynes JW (1998) Immunohistochemical and ELISA assays for biomarkers of oxidative stress in aging and disease. *Ann NY Acad Sci* 854: 277–290.
6. Daimon M, Sugiyama K, Kameda W, Saitoh T, Oizumi T, Hirata A, Yamaguchi H, Ohnuma H, Igarashi M, Kato T (2003) Increased urinary levels of pentosidine, pyrrole and acrolein adduct in type 2 diabetes. *Endocr J* 50: 61–67.
7. Ono Y, Aoki S, Ohnishi K, Yasuda T, Kawano K, Tsukada Y (1998) Increased serum levels of advanced glycation end-products and diabetic complications. *Diabetes Res Clin Pr* 41: 131–137.
8. Sanaka T, Funaki T, Tanaka T, Hoshi S, Niwayama J, Taitoh T, Nishimura H, Higuchi C (2002) Plasma pentosidine levels measured by a newly developed method using ELISA in patients with chronic renal failure. *Nephron* 91: 64–73.
9. Monnier VM, Bautista O, Kenny D, Sell DR, Fogarty J, Dahms W, Cleary PA, Lachin J, Genuth S, the DCCT Skin Collagen Ancillary Study Group (1999) Skin collagen glycation, glycoxidation, and crosslinking are lower in subjects with long-term intensive versus conventional therapy of type 1 diabetes. *Diabetes* 48: 870–880.
10. Baynes JW, Thorpe SR (1999) Role of oxidative stress in diabetic complications. A new perspective on an old paradigm. *Diabetes* 48: 1–9.
11. Miyata T, Maeda K, Kurokawa K, van Ypersele de Strihou C (1997) Oxidation conspires with glycation to generate noxious advanced glycation end products. *Nephrol Dial Transplant* 12: 255–258.
12. Dyer DG, Blackledge JA, Thorpe SR, Baynes JW (1991) Formation of pentosidine during nonenzymatic browning of protein by glucose: Identification of glucose and other carbohydrates as possible precursors of pentosidine in vivo. *J Biol Chem* 266: 11654–11660.
13. Sell DR, Lapolla A, Odetti P, Fogarty J, Monnier VM (1992) Pentosidine formation in skin correlates with severity of complications in individuals with long standing IDDM. *Diabetes* 41: 1286–1292.
14. Suzuki D, Miyata T, Saotome N, Horie K, Inagi R, Yasuda Y, Uchida K, Izuhara Y, Yagame M, Sakai H, Kurokawa K (1999) Immunohistochemical evidence for an increased oxidative stress and carbonyl modification of proteins in diabetic glomerular lesions. *J Am Soc Nephrol* 10: 822–832.
15. Takeuchi M, Makita Z, Yanagisawa K, Kameda Y, Koike T (1999) Detection of noncarboxymethyllysine and carboxymethyllysine advanced glycation end products (AGE) in serum of diabetic patients. *Mol Med* 5: 393–405.
16. Endo M, Yanagisawa K, Tsuchida K (2001) Increased levels of vascular endothelial growth factor and advanced glycation end products in aqueous humor of patients with diabetic retinopathy. *Horm Metab Res* 33: 317–322.
17. McCance DR, Dyer DG, Dunn JA (1993) Maillard reaction products and their relation to complications in insulin-dependent diabetes mellitus. *J Clin Invest* 91: 2470–2478.
18. Beisswenger PJ, Moore LL, Truls BJ, Curphey TJ (1993) Increased collagen-linked pentosidine and advanced glycosylation end products in early diabetic nephropathy. *J Clin Invest* 92: 212–217.
19. Tanji N, Markowitz GS, Fu C (2001) Expression of advanced glycation end products and their cellular receptor RAGE in diabetic nephropathy and nondiabetic renal disease. *J Am Soc Nephrol* 11: 1656–1666.

20. Vlassala H, Striker LJ, Teichberg S, Fuh H, Li YM, Steffes M (1994) Advanced glycation end products induce glomerular sclerosis and albuminuria in normal rats. *P Natl Acad Sci USA* 91: 11704–11708.
21. Kiuchi K, Nejima J, Takano T, Ohta M, Hashimoto H (2001) Increased serum concentrations of advanced glycation end products: a marker of coronary artery disease activity in type 2 diabetic patients. *Heart* 85: 87–91.